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74 063

From: Slobodyansky, Elizabeth
Sent: Monday, September 02, 2002 5:17 PM
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Subject: 09/719,017

Please search for case 09/719,017:

ES

SEQ ID NOs: 1 and 2 against commercail and interference databases.

Thank you.

Elizabeth Slobodyansky, PhD

Primary Examiner

Art Unit 1652
CM1 10D11
703-306-3222

mail box 10C01

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Searcher: _____
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Online time: _____

TYPE OF SEARCH:
NA Sequences: _____
AA Sequences: _____
Structures: _____
Bibliographic: _____
Litigation: _____
Full text: _____
Patent Family: _____
Other: _____

VENDOR/COST (where applic.)
STN: _____
DIALOG: _____
Questel/Orbit: _____
DRLink: _____
Lexis/Nexis: _____
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WWW/Internet: _____
Other (specify): _____

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OM nucleic - nucleic search, using sw model

Run on: September 7, 2002, 22:27:06 ; Search time 1772.12 Seconds
(without alignments)
921.569 Million cell updates/sec

Title: US-09-719-017A-1

Sequence: 1 gaattccctgtgacaatta.....tatctaaggaataacttaca 121

Scoring table: IDENTITY NUC
Gapop 10.0, Gapext 1.0

Searched: 13736207 seqs, 6748477542 residues

Total number of hits satisfying chosen parameters: 27472414

Minimum DB seq length: 0

Maximum DB seq length: 200000000

Post-processing: Minimum Match 0%

Maximum Match 100%
Listing first 45 summaries

Database :

EST:*
1: em_estba:*
2: em_esthum:*
3: em_estin:*
4: em_estmu:*
5: em_estov:*
6: em_estpl:*
7: em_estro:*
8: em_hic:*
9: gb_estl:*
10: gb_est2:*
11: gb_hic:*
12: gb_gss:*
13: em_gss_hum:*
14: em_gss_inv:*
15: em_gss_pln:*
16: em_gss_vrti:*

Pred. No. is the number of results predicted by chance to have a score greater than or equal to the score of the result being printed, and is derived by analysis of the total score distribution.

SUMMARIES

Result No.	Score	Query Match	Length	DB ID	Description
1	41.4	34.2	719	12	AG010512 Homo sapi
2	40.6	33.6	516	10	BE434941 EST406019
3	40.6	33.6	517	10	BE434941 EST406019
4	40.6	33.6	520	10	BE434941 EST406019
5	40.6	33.6	564	10	BE434941 EST406019
6	40.6	33.6	575	9	AV404501 AV404501
7	40.6	33.6	579	10	BE434941 EST406019
8	40.6	33.6	643	9	AL507443 AL507443
9	40.6	33.6	677	10	BE434941 EST406019
10	40.6	33.6	685	9	AV403776 AV403776
11	40.6	33.6	688	9	AV170489 AV170489
12	40.6	33.6	722	10	BF657631 BF657631
13	40.6	33.6	774	12	AG002309 Homo sapi
14	40.6	33.6	486	10	BJ029323 BJ029323
15	36.4	29.8	412	9	AU023129 AU023129
16	36.4	29.8	412	9	AG010513 Homo sapi
17	35.6	29.4	543	10	W26134 W26134

C 18	35.2	29.1	232	9	BB064396
C 19	35.2	29.1	429	9	AV268865
C 20	35.2	29.1	429	9	BB713280
C 21	35.2	29.1	481	10	BB068519
C 22	33.8	27.9	551	12	BB460835
C 23	32.8	27.1	1101	12	CNS016TC
C 24	32.6	26.9	611	9	AV386177
C 25	32	26.4	751	12	AG002318
C 26	31.6	26.1	820	12	BB269175
C 27	31	25.6	1077	10	BB198012
C 28	30.4	25.1	261	9	BB215029
C 29	30.4	25.1	592	9	AI526051
C 30	29.6	24.5	430	12	AA297677
C 31	29.6	24.5	1101	12	CNS051TC
C 32	29.4	24.3	911	12	AA680228
C 33	29.4	24.3	960	12	BB152788
C 34	29.4	24.3	980	12	BB151047
C 35	29.2	24.1	551	12	TA18902P
C 36	29.2	24.0	920	12	CNS06VMU
C 37	29	24	517	12	AA069512
C 38	28.8	23.8	959	12	CNS06V7B
C 39	28.6	23.6	958	12	CNS00M48
C 40	28.4	23.5	469	12	AA0769049
C 41	28.4	23.5	520	12	AA0943779
C 42	28.4	23.5	541	9	AA633688
C 43	28.4	23.5	632	9	BB318353
C 44	28.2	23.3	503	12	BB9571
C 45	28.2	23.3	508	12	BB110380

ALIGNMENTS

RESULT 1
LOCUS AG010512/c 719 bp DNA linear GSS 14-APR-1999
DEFINITION Homo sapiens genomic DNA, 21q region, clone: Q89A6SPN6, genomic survey sequence.
ACCESSION AG010512 AG004386
VERSION AG010512.1 GI:3327331
KEYWORDS GSS.
SOURCE Homo sapiens DNA, clone: Q89A6SPN6.
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Homnidae; Homo.
REFERENCE Hattori, M., Ishii, K., Toyoda, A., Shiba, T. and Sakaki, Y.
TITLE Published Only in Database (1998) In press
AUTHORS Hattori, M., Ishii, K., Toyoda, A., Shiba, T. and Sakaki, Y.
TITLE Direct Submission
JOURNAL Submitted (16-JUL-1998) to the DDBJ/EMBL/GenBank databases.
Masahira Hattori, Kitasato University, Department of Science, JST Sequencing Laboratory, Kitasato I-15-1, Sagamihara 228, Japan (E-mail: hattori@hgc.ims.u-tokyo.ac.jp, Tel: 0427-78-9732, Fax: 0427-78-9561)
COMMENT On Feb 5, 1999 this sequence version replaced gi:2789521.
AG004386: Submitted (17-Jan-1999).
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/clone="Q89A6SPN6"
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BASE COUNT 167 a 171 c 159 g 218 t 4 others
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Matches 45; Conservative 0; Mismatches 6; Indels 0; Gaps 0;

QY 71 gccacgctgggcatacattcaatctgttcttaaggaatacttaca 121
|||||
Db 513 GCCATTATTGCATCATTCATCAATGTTATCTAAGGAATACTTACA 463

RESULT 2
LOCUS BE34941/C 516 bp mRNA linear EST 18-MAY-2001
DEFINITION EST406019 tomato breaker fruit, TIGR Lycopersicon esculentum cDNA
ACCESSION BE34941
VERSION BE34941.1 GI:9432784
KEYWORDS EST.
SOURCE tomato.
ORGANISM Lycopersicon esculentum
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
Asterales; eustersids I; Solanales; Solanaceae; Solanum;
Lycopersicon.
1 (bases 1 to 516)
Alcala, J., Vrebalov, J., White, R., van der Hoeven, R.S., Holt, I.E.,
Liang, F., Hansen, T.S., Craven, M.B., Bowman, C.L., Ronning, C.M.,
Niernmen, W., Fraser, C.M., Martin, G.B., Giovannoni, J.J. and Tanksley
S.D.
Generation of ESTs from tomato fruit tissue, breaker stage
Unpublished (2000)
Contact: CUGI
Clemson University Genomics Institute
Clemson University
100 Jordan Hall, Clemson, SC 29634, USA
Email: <http://www.genome.clemson.edu/orders/index.html>
5 prime sequence.
Location/Qualifiers
1. 516
/organism="Lycopersicon esculentum"
/cultivar="T496"
/db_xref="taxon:4081"
/clone="CLEG2412"
/clone_lib="tomato breaker fruit, TIGR"
/issue_type="pericarp"
/dev_stage="breaker"
/lab_host="SOLR"
/note="Vector: pBluescriptSkmcUnadapt; Site_1: EcoRI;
Site_2: XhoI; Fruit were harvested at the breaker stage
(first sign of lycopene accumulation on the blossom end of
the fruit). Fruit were cut in half and the seeds and
locules were discarded prior to freezing the pericarp."

BASE COUNT 128 a 112 c 106 g 170 t

ORIGIN

Query Match 33.6%; Score 40.6; DB 10; Length 516;
Best Local Similarity 83.6%; Pred. No. 0.006;
Matches 46; Conservative 0; Mismatches 9; Indels 0; Gaps 0;

QY 67 tgcagccaagctgggcatacattcaatctgttcttaaggaatacttaca 121
|||||
Db 175 TGTATGCATTTATTTCATCATTCATCAATGTTATCTAAGGAATACTTACA 121

RESULT 3
LOCUS BI269359 517 bp mRNA linear EST 18-JUL-2001
DEFINITION NF008A071R1052 Irradiated Medicago truncatula cDNA clone
ACCESSION BI269359
VERSION BI269359.1 GI:14876004
KEYWORDS EST.
SOURCE barrel medic.
ORGANISM Medicago truncatula
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;

Posidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Trifoliaceae;
Medicago.
1 (bases 1 to 517)
Torres-Jerez, I., Scott, A.D., Harris, A.R., Gonzales, R.A., Bell, C.J.,
Flores, H.R., Imman, J.T., Weller, J.W. and May, G.D.
Expressed Sequence Tags from the Samuel Roberts Noble Foundation
Medicago truncatula irradiated library
Unpublished (2001)
Contact: May GD
Plant Biology Division
The Samuel Roberts Noble Foundation
2510 Sam Noble Parkway, Ardmore, OK 73402, USA
Tel: 580 221 7391
Fax: 580 221 7380
Email: gdmay@noble.org
Insert Length: 517 Std Error: 0.00
Plate: 008 row: A column: 07
Seq primer: TCACACGAGAAACGCTATGAC.
Location/Qualifiers
1. 517
/organism="Medicago truncatula"
/db_xref="taxon:3880"
/clone="NF008A071R"
/clone_lib="Irradiated"
/issue_type="seedlings"
/dev_stage="seedling"
/note="Vector: Lambda Zap; Seedlings were exposed either
to 100 Gy gamma or 0.5, 1, 5, or 10 kJ/m2 UV irradiation.
Gamma-irradiated samples were harvested at 6, 12, 24 and
48 hours after treatment. UV-irradiated samples were
harvested 24 hours post-treatment. cDNA was prepared from
polyA+ enriched, pooled samples of equivalent amounts of
total RNA from each sample. The cDNA was directionally
ligated into the Uni-Zap XR vector (Stratagene) and
packaged using the Gigapack III Gold packaging extracts.
Phagemids containing cDNA inserts were in vivo excised
from the recombinant Uni-Zap XR vector using Exsist
helper phage and the E. coli strain XL1-Blue MRP.
(Stratagene). Excised plasmids were plated using SOLR
cells."

BASE COUNT 161 a 114 c 122 g 119 t 1 others

ORIGIN

Query Match 33.6%; Score 40.6; DB 10; Length 517;
Best Local Similarity 83.6%; Pred. No. 0.006;
Matches 46; Conservative 0; Mismatches 9; Indels 0; Gaps 0;

QY 67 tgcagccaagctgggcatacattcaatctgttcttaaggaatacttaca 121
|||||
Db 200 TGTATGCATTTATTTCATCATTCATCAATGTTATCTAAGGAATACTTACA 254

RESULT 4
LOCUS BI269270 520 bp mRNA linear EST 18-JUL-2001
DEFINITION NF005G121R1099 Irradiated Medicago truncatula cDNA clone
ACCESSION NF005G121R 5', mRNA sequence.
VERSION BI269270
KEYWORDS EST.
SOURCE barrel medic.
ORGANISM Medicago truncatula
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Trifoliaceae;
Medicago.
1 (bases 1 to 520)
Torres-Jerez, I., Scott, A.D., Harris, A.R., Gonzales, R.A., Bell, C.J.,
Flores, H.R., Imman, J.T., Weller, J.W. and May, G.D.
Expressed Sequence Tags from the Samuel Roberts Noble Foundation
Medicago truncatula irradiated library
Unpublished (2001)

COMMENT	Contact: May GD Plant Biology Division The Samuel Roberts Noble Foundation 2510 Sam Noble Parkway, Ardmore, OK 73402, USA Tel: 580 221 7391 Fax: 580 221 7380 Email: gdmay@noble.org Insert Length: 520 Std Error: 0.00 Plate: 005 row: 6 column: 12 Seq primer: TCACACAGAAACGCTATGCAC. Location/Qualifiers
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	/organism="Medicago truncatula" /db_xref="taxon:3880" /clone="NF005G12IR" /clone_1lb="Irradiated" /_lssue_type="Seedlings" /_dev_stage="seedling" /_note="Vector: Lambda Zap; Seedlings were exposed either to 100 Gy gamma or 0.5, 1, 5, or 10 kJ/m2 UV irradiation. Gamma-irradiated samples were harvested at 6, 12, 24 and 48 hours after treatment. UV-irradiated samples were harvested 24 hours post-treatment. cDNA was prepared from polyA+ enriched, pooled samples of equivalent amounts of total RNA from each sample. The cDNA was directionally ligated into the Uni-Zap XR vector (Stratagene) and packaged using the Gigapack III Gold packaging extracts. Phagemids containing cDNA inserts were in vivo excised from the recombinant Uni-ZAP XR vector using EXASist helper phage and the E. coli strain XL1-Blue MRF' (Stratagene). Excised plasmids were plated using SOLR cells."
BASE COUNT	162 a 114 c 124 g 119 t 1 others
ORIGIN	
Query Match	33.6%; Score 40.6; DB 10; Length 520;
Best Local Similarity	83.6%; Pred. No. 0.006;
Matches	46; Conservative 0; Mismatches 9; Indels 0; Gaps 0;
Oy	67 tgcacccaagtctggcgcatcacatcaatgtgattctaagaataacttaca 121 Db 200 TGTATGCATTATTTCGACATCATTCATCAATCATTATCTTAAGGAATACTTACA 254
RESULT	5
BE460888	564 bp mRNA linear EST 18-MAY-2001
LOCUS	ESN412307 tomato breaker fruit, TIGR Lycopersicon esculentum cDNA
DEFINITION	EST412307 tomato breaker fruit, TIGR Lycopersicon esculentum cDNA clone CLEB36H10, mRNA sequence.
ACCESSION	BE460888
VERSION	BE460888.1 GI:9505190
KEYWORDS	EST.
SOURCE	tomato.
ORGANISM	Lycopersicon esculentum Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Asterales; euasterids I; Solanales; Solanaceae; Solanum; Lycopersicon.
REFERENCE	1 (bases 1 to 564) Alcalai,J., Vrebalov,J., White,R., van der Hoeven,R.S., Holt,I.E., Liang,F., Hansen,T.S., Craven,M.B., Bowman,C.L., Roming,C.M., Niernman,W., Fraser,C.M., Martin,G.B., Giovannoni,J.J. and Tanksley
AUTHORS	'S.D.
TITLE	Generation of ESTs from tomato fruit tissue, breaker stage
JOURNAL	Unpublished (2000)
COMMENT	Contact: CUGI Clemson University Genomics Institute Clemson University 100 Jordan Hall, Clemson, SC 29634, USA Email: http://www.genome.clemson.edu/orders/index.html 5 prime sequence. Location/Qualifiers
FEATURES	

	source	1..564	/organism="Lycopersicon esculentum"
			/cultivar="TA496"
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			/clone="cLEG3H10"
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			/tissue_type="pericarp"
			/dev_stage="breaker"
			/lab_host="SOLR"
			/note="Vector: pluscriptSKMcnadpt; Site_1: EcorI;
			Site_2: XhoI; Fruit were harvested at the breaker stage
			(first sign of lycopene accumulation on the blossom end
			of the fruit). Fruit were cut in half and the seeds and
			locules were discarded prior to freezing the pericarp."
BASE COUNT		173 a 123 c 135 g 133 t	
ORIGIN			
Query Match		33.6%; Score 40.6; DB 10; Length 564;	
Best Local Similarity		83.6%; Pred. No. 0.0062;	
Matches	46; Conservative	0; Mismatches 9; Indels 0; Gaps 0;	
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Dd	74	TGTATGCAATTATTTGGCATTCATTCATCAATGTTATCTAAGAAATCTTACA 128	
RESULT	6		
LOCUS	AV404501	575 bp mRNA linear EST 06-FEB-2000	
DEFINITION	AV404501 Bombyx mori prothoracic gland p50 5th-instar day-4 larva		
ACCESSION	AV404501		
VERSION	AV404501.1	GI:6908589	
KEYWORDS	EST.		
SOURCE	Bombyx mori		
ORGANISM	domestic silkworm.		
	Eukaryota; Metazoa; Arthropoda; Tracheata; Hexapoda; Insecta;		
	Pterygota; Neoptera; Endopterygota; Lepidoptera; Glossata; Ditrysia		
	; Bombycoidea; Bombycidae; Bombyx.		
REFERENCE	1 (bases 1 to 575)		
AUTHORS	Mita,K., Morimyo,M., Shimada,T., Okano,K. and Maeda,S.		
TITLE	Bombyx mori cDNA		
JOURNAL	Unpublished (2000)		
COMMENT	Contact: Mita K Genome Research Group National Institute of Radiological Sciences Anagawa 4-9-1, Inage, Chiba 263-8555, Japan Email: kmिता@irs.go.jp method:uni-directional, sequence direction:sequenced from T3 primer (5' -> 3')		
FEATURES	Project-"Silkorm Genome Program in MARF, and Research for the Future Program in JSPS". see "SilkBase" < http://www.ab.a.u-tokyo.ac.jp/silkbase/ >, for whole ESTdb. Location/Qualifiers		
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	day-4 larva"		
	/sex="female/male mixed"		
	/tissue_type="prothoracic gland"		
	/dev_stage="5th-instar day-4 larva"		
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Query Match		33.6%; Score 40.6; DB 9; Length 575;	
Best Local Similarity		83.6%; Pred. No. 0.0062;	
Matches	46; Conservative	0; Mismatches 9; Indels 0; Gaps 0;	

- site. *Nucleic Acids Res.* 11:5837-5854.
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encoding genes or by methods that activate a large battery of chaperone molecules in the cell.

The second is the realization of a "true" and robust secretion mechanism for the efficient release of protein into the culture medium. There are several available systems that facilitate secretion of recombinant proteins into the culture medium. Some of these are based on the use of signal peptides, fusion partners, and permeabilizing agents that cause disruption and limited leakage of the outer membrane. Other efforts are directed at pirating existing secretion pathways that promise greater specificity of secretion. Work in this area will necessitate an improved understanding of the various secretion pathways in *E. coli*.

The third is the endowment of the prokaryotic cell with the ability to perform some of the posttranslational modifications found in eukaryotic proteins, such as glycosylation. This might be done by the engineering of eukaryotic glycosylating enzymes into the *E. coli* chromosome.

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culture medium (reference 284 and references therein). Nutrient composition and fermentation variables such as temperature, pH and other parameters can affect proteolytic activity, secretion, and production levels (24, 25, 153, 324, 338, 614). Specific manipulations of the culture medium have been shown to enhance protein release into the medium. Thus, supplementation of the growth medium with glycine enhances the release of periplasmic proteins into the medium without causing significant cell lysis (10, 13). Similarly, growth of cells under osmotic stress in the presence of sorbitol and glycyl betaine causes more than a 400-fold increase in the production of soluble, active protein (49).

High-cell-density culture systems suffer from several drawbacks, including limited availability of dissolved oxygen at high cell density, carbon dioxide levels which can decrease growth rates and stimulate acetate formation, reduction in the mixing efficiency of the fermentor, and heat generation. The techniques that are used to minimize such problems have been examined in detail (338). A major challenge in the production of recombinant protein at high cell density is the accumulation of acetate, a lipophilic agent that is detrimental to cell growth (285, 338, 353). A number of strategies have been developed to reduce acetate formation in high-cell-density cultures, but these suffer from several drawbacks (338). This problem was recently resolved through the metabolic engineering of *E. coli* (11, 12, 479). The *alsS* gene from *B. subtilis* encoding the enzyme acetolactate synthase was introduced into *E. coli* cells. This enzyme catalyzes the conversion of pyruvate to nonacidic and less toxic byproducts. The reduction in acetate accumulation caused a significant improvement in the production of recombinant protein (12, 479). Mutant strains of *E. coli* that are deficient in other enzymes have also been developed and shown to produce less acetate and higher levels of human recombinant proteins (30, 103, 273).

CONCLUSIONS AND FUTURE DIRECTIONS

An efficient prokaryotic expression vector should contain a strong and tightly regulated promoter, an SD site that is positioned approximately 9 bp 5' to the translation initiation codon and is complementary to the 3' end of 16S rRNA, and an efficient transcription terminator positioned 3' to the gene coding sequence. In addition, the vectors require an origin of replication, a selection marker, and a gene that facilitates the stringent regulation of promoter activity. This regulatory element may be integrated either in the vector itself or in the host chromosome. Other elements that may be beneficial include transcriptional and translational "enhancers," as well as "minicistrons" in translationally coupled systems. These may be gene specific; therefore, their utility must be tested case by case. The translational initiation region of a gene must be free of secondary structures that may occlude the initiation codon and/or block ribosome binding. UAAU is the most efficient translation termination sequence in *E. coli*.

There are many different prokaryotic vectors that allow the tight regulation of gene expression. The experimental approaches to achieve tight regulation of promoter activity range from the simple repositioning of the operator in *lac*-based systems to the construction of elaborate "cross-regulation" systems. These vectors are efficient, and each system has its own niche in prokaryotic gene expression. The demonstrated effectiveness of a thermosensitive *lac* repressor now allows the thermal regulation of *lac*-based promoters in lieu of using IPTG.

To date, there is no generally applicable strategy to prevent the degradation of a wide variety of mRNA species in *E. coli*.

Although certain 5' and 3' stem-loop structures have been shown to block mRNA degradation, these seem to stabilize only specific mRNAs, under restricted conditions. One exception appears to be the 5' UTR of the *E. coli ompA* transcript, which prolongs the half-life of a number of heterologous mRNAs in *E. coli*. The use of strains deficient in specific RNases has been ineffective for enhanced gene expression.

Each of the four "compartments" for targeted protein production, i.e., the cytoplasm, periplasm, inner and outer membranes, and growth medium, offers advantages and disadvantages for gene expression, depending on the experimental objectives. The formation of inclusion bodies can be minimized by a variety of techniques, but it remains a significant barrier to high-level protein production in the cytoplasm. To date, the effectiveness of molecular chaperones has been protein specific. It is possible that this is due to conditions that prevent the formation of a thermodynamically stable end product, such as the production of severely truncated proteins or single domains from multisubunit protein complexes, lack of formation of disulfide bonds, suboptimal growth conditions, absence of posttranslational modifications, and the normally concerted action of multiple types of chaperones in vivo. Nevertheless, molecular chaperones have been used very successfully for the enhanced production of specific proteins.

The wide variety of existing fusion partners have utility in the production, detection, and purification of recombinant proteins. Specific fusion moieties can increase the folding, solubility, resistance to proteolysis, and secretion of recombinant proteins into the growth medium.

Protein misfolding, attributed to the intracellular concentration of aggregation-prone intermediates, may be minimized by a combination of experimental approaches: replacement of amino acid residues that cause aggregation, coexpression of molecular chaperones and foldases, reduction of the rate of protein synthesis, the use of solubilizing fusion partners, and the careful optimization of growth conditions.

Codon usage can have adverse effects on the synthesis and yield of recombinant proteins. However, the mere presence of "rare" codons in a gene does not necessarily dictate poor translation of that gene. Currently, we do not know all the rules that link codon usage and translation of a transcript. The lack of consistent results in the published literature on codon usage may be due to several variables, such as positional effects, the clustering or interspersing of the rare codons, secondary structure of the mRNA, and other effects. Positional effects appear to play an important role in protein synthesis. Thus, the presence of rare codons near the 5' end of a transcript probably affects translational efficiency. This problem may be rectified by the alteration of the culprit codons, and/or the coexpression of the cognate tRNA genes.

Much progress has been made in the elucidation of specific determinants of protein degradation in *E. coli*. Effective approaches for the minimization of proteolysis in *E. coli* include the targeting of protein to the periplasm or the culture medium, the use of protease-deficient host strains, the construction of fusion proteins, the coexpression of molecular chaperones, the coexpression of the T4 *pin* gene, the elimination of protease cleavage sites through genetic engineering, and the optimization of fermentation conditions. Host strains that are deficient in the *ipoH* (*hupR*) locus are among the best, particularly for thermally induced expression systems.

Future challenges in the use of *E. coli* for gene expression will involve the following factors. The first is the achievement of enhanced yields of correctly folded proteins by manipulating the molecular chaperone machinery of the cell. Perhaps this might be done by the coexpression of multiple chaperone-

was recently discovered (297) and a fascinating new mechanism for the degradation of abnormal proteins in *E. coli* has just been uncovered (299). Nevertheless, the intense scientific interest in this area has generated new tools and strategies for minimizing the degradation of heterologous proteins in *E. coli*.

Although the precise structural features that impart lability to proteins are not known, some determinants of protein instability have been elucidated. In a series of systematic studies, Varshavsky and colleagues formulated the "N-end rule" that relates the metabolic stability of a protein to its amino-terminal residue (14, 15, 209, 560, 571). Thus, in *E. coli*, N-terminal Arg, Lys, Leu, Phe, Tyr, and Trp conferred 2-min half-lives on a test protein, whereas all the other amino acids except proline conferred more than 10-h half-lives on the same protein (560). As discussed above (see the section on cytoplasmic expression), amino acids with small side chains in the second position of the polypeptide facilitate the methionine aminopeptidase catalyzed removal of the N-terminal methionine (250). Therefore, these studies suggest that Leu in the second position would probably be exposed by the removal of the methionine residue and would destabilize the protein.

The second determinant of protein instability is a specific internal lysine residue located near the amino terminus (14, 15, 86). This residue is the acceptor of a multiubiquitin chain that facilitates protein degradation by a ubiquitin-dependent protease in eukaryotes. Interestingly, in a multisubunit protein, the two determinants can be located on different subunits and still target the protein for processing (287).

Another correlation between amino acid content and protein instability is presented in the PEST hypothesis (461). On the basis of statistical analysis of eukaryotic proteins that have short half-lives, it was proposed that proteins are destabilized by regions enriched in Pro, Glu, Ser, and Thr, flanked by certain amino acid residues. Phosphorylation of these PEST domains leads to increased calcium binding, which in turn facilitates the destruction of the protein by calcium-dependent proteases. It was suggested that PEST-rich proteins may be produced efficiently in *E. coli*, which apparently lacks the PEST proteolytic system (461).

Strategies for minimizing proteolysis of recombinant proteins in *E. coli* have been reviewed in detail (25, 153, 395) and are summarized in Table 2. These include protein targeting to the periplasm (550) or the culture medium (230), the use of protease-deficient host strains (211), growth of the host cells at low temperature (100), construction of N- and/or C-terminal fusion proteins (59, 230, 319, 393), tandem fusion of multiple copies of the target gene (512), coexpression of molecular chaperones (489, 581), coexpression of the T4 *pin* gene (519–521), replacement of specific amino acid residues to eliminate protease cleavage sites (243), modification of the hydrophobicity of the target protein (394), and optimization of fermentation conditions (24, 338).

Although the variety of approaches for protein stabilization attests to the ingenuity of the investigators, the usefulness of some of the above methods may be limited, depending on the intended use of the recombinant protein. Thus, for example, the presence of fusion moieties on the target protein may interfere with functional or structural properties (51) or therapeutic applications of the product. The engineering of enzymatic or chemical cleavage sites for the subsequent removal of the fusion partners is a complex process that involves numerous considerations: the accessibility of the cleavage sites to enzyme digestion; the purity, specificity, and cost of the commercially available enzymes; the authenticity of the N or C termini upon enzymatic digestion; the possible modification of the target protein upon chemical treatment, and so forth (see,

e.g., references 78, 162, 419, and 567). For the large-scale production of fusion proteins, some of these difficulties are amplified. Similarly, the fusion of multiple copies of the target gene to create multidomain polypeptides (512) requires the subsequent conversion to monomeric protein units by cyanogen bromide cleavage. In this case, the target protein must not contain internal methionine residues and must be able to withstand harsh reaction conditions. Moreover, a limited extent of amino acid side chain modification may occur, and the toxicity of cyanogen bromide presents a significant issue for large-scale cleavage reactions. Similarly, the rational modification of a protein sequence requires extensive structural information which may not be available. Molecular chaperones have been used successfully to stabilize specific proteins (395), but this approach remains a hit-or-miss affair (581).

The cytoplasm of *E. coli* contains a greater number of proteases than does the periplasm (545, 546). Therefore, proteins located in the periplasm are less likely to be degraded. For example, proinsulin localized to the periplasm was 10-fold more stable than when produced in the cytoplasm (550). However, proteolytic activity in the periplasm is substantial (367). Secretion into the culture medium would provide a better alternative in terms of protein stability. Unfortunately, the technology for secretion of proteins from *E. coli* into the culture medium is still in its infancy (528) (see the section on extracellular secretion, above). A major catalyst of protein degradation in bacteria is the induction of heat shock proteins in response to a variety of stress conditions, such as the thermal induction of gene expression or the accumulation of abnormal or heterologous proteins in the cytoplasm (194). Under these conditions, the production of the *lon* gene product, protease La (195), and other proteases is enhanced. This problem is minimized by the use of host strains deficient in the *rpoH* (*hipR*) locus (201, 211, 421). The *rpoH* gene encodes the RNA polymerase σ^{32} subunit, which regulates several proteolytic activities in *E. coli* (20, 193). Hosts that carry the *rpoH* mutation have been patented (202) and have been demonstrated to dramatically increase the production of foreign proteins in *E. coli* (see, e.g., references 4, 9, 47, 70, and 373). Strain SG21173 (211), which is deficient in proteases La and Clp and the *rpoH* locus, is particularly effective in protein production (9). A large number of protease-deficient hosts exists (see, e.g., references 23 and 211), including some that are deficient in all known protease loci that affect the stability of secreted proteins (372).

Before leaving this section, it is worth repeating a caveat on the use of protease-deficient strains (581): proteolysis may be an effect rather than a cause of folding problems, serving as a disposal system to remove misfolded and aggregated material (238). Therefore, it is possible that the absence of proteases will result in increased toxicity to the host as a result of the accumulation of abnormal proteins.

FERMENTATION CONDITIONS

Protein production in *E. coli* can be increased significantly through the use of high-cell-density culture systems, which can be classified into three groups: batch, fed batch, and continuous. These methods can achieve cell concentrations in excess of 100 g (dry cell weight)/liter and can provide cost-effective production of recombinant proteins. Detailed reviews of large-scale fermentation systems have been published (338, 607, 614). The composition of the cell growth medium must be carefully formulated and monitored, because it may have significant metabolic effects on both the cells and protein production. For example, the translation of different mRNAs is differentially affected by temperature as well as changes in the

TABLE 4. Low-usage codons in *E. coli*^a

Codon(s)	Amino acid
AGA, AGG, CGA, CGG	Arg
UGU, UGC	Cys
GGA, GGG	Gly
AUA	Ile
CUA, CUC	Leu
CCC, CCU, CCA	Pro
UCA, AGU, UCG, UCC	Ser
ACA	Thr

^a The reported frequency of codon usage varies depending on the author (based on references 293, 580, and 623).

the *E. coli* periplasm is facilitated by a group of proteins that maintain the correct redox potential (26). It is thought that DsbA, a soluble periplasmic protein, directly catalyzes disulfide bond formation in proteins whereas DsbB, an inner membrane protein, is involved in the reoxidation of DsbA (227a). Eukaryotic PDI was capable of complementing the phenotypes of *dsbA* null mutants (268, 433a), but its function was virtually abolished in *dsbB* mutants (433a). In addition, the ability of PDI to enhance the yield of target proteins was increased in the presence of exogenously added glutathione (268, 433a). These observations suggest that PDI depends on the presence of bacterial redox proteins for its reoxidation. The coexpression of rat PDI has also been reported to enhance the correct folding of tissue plasminogen activator (433a).

Protein misfolding can be attributed to the intracellular concentration of aggregation-prone intermediates. Thus, although the subject of this review is the maximization of protein synthesis, reducing the rate of protein synthesis should disfavor protein misfolding. Indeed, the use of weaker promoters or conditions of partial induction from stronger promoters can result in larger amounts of soluble protein (180, 253). Kadokura et al. (291) showed that the ability of *E. coli* mutants to secrete a large amount of alkaline phosphatase into the periplasm was due to a lower synthetic rate of the *phoA* gene product.

CODON USAGE

Genes in both prokaryotes and eukaryotes show a nonrandom usage of synonymous codons (214, 228, 272, 509, 623). The systematic analysis of codon usage patterns in *E. coli* led to the following observations (124). (i) There is a bias for one or two codons for almost all degenerate codon families. (ii) Certain codons are most frequently used by all different genes irrespective of the abundance of the protein; for example, CCG is the preferred triplet encoding proline. (iii) Highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones. (iv) The frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs. These observations imply that heterologous genes enriched with codons that are rarely used by *E. coli* (Table 4) may not be expressed efficiently in *E. coli*.

The minor arginine tRNA^{Arg} (AGG:AGA) has been shown to be a limiting factor in the bacterial expression of several mammalian genes (62), because the codons AGA and AGG are infrequently used in *E. coli* (91, 95, 214). The coexpression of the *argU* (*dnaY*) gene that codes for tRNA^{Arg} (AGG:AGA) (175, 343) resulted in high-level production of the target protein (62). The production of β -galactosidase decreased when AGG codons were inserted before the 10th codon from the initiation codon of the *lacZ* gene (92). Similarly, Goldman et al. (204)

reported that translational inhibition of a test mRNA was much stronger in both arginine and leucine cases when the consecutive low-usage codons were located near the 5' end of the mRNA. Ivanov et al. (280) reported that tandem AGG triplets caused a substantial inhibition of gene expression independent of their localization in mRNA. These workers attributed the inhibitory effect to a competition of the tandem AGGAGG codons with the natural SD sequence. Other studies showed that protein production levels could be increased either by substitution of high-usage codons for low-usage ones (see, e.g., references 3, 70, 135, 145, 248, 262, 383, 452) or by coexpression of the "rare" tRNA gene (62, 126). The expression of the ICP4 gene from herpes simplex virus was shown to be inefficient because of the presence of an almost continuous stretch of 19 serine residues (73). The efficiency of ICP4 synthesis was not improved by silent mutations in this serine-rich region, supplementation of the growth medium with serine, overexpression of seryl-tRNA synthetase, or expression of tRNA^{Ser5}. The level of gene expression was inversely proportional to the number of serine codons in this region (73). Although this is certainly an extreme case, it is indicative of the adverse effects of long stretches of similar codons on translational efficiency.

In contrast, other workers reported very efficient expression of genes that contained low-usage codons (see, e.g., references 154, 265, 334, 464, and 616). Similarly, in the case of the human T-cell receptor V β 5.3 gene that contains 4% AGA/AGG codons, expansion of the intracellular pool of tRNA^{Arg} (AGG:AGA) did not significantly increase the amount of V β 5.3 detected in the cells (9).

The evolutionary significance of codon usage patterns, as well as mechanistic explanations for the effects of codon usage, has been advanced by many workers (74, 92, 124, 155, 204, 245, 276, 293, 463, 474). To date, however, it has not been possible to formulate general and unambiguous "rules" to predict whether the content of low-usage codons in a specific gene might adversely affect the efficiency of its expression in *E. coli*. The experimental results may be confounded by several variables, such as positional effects, the clustering or interspersal of the rarely used codons, the secondary structure of the mRNA, and other effects (204, 293). Nevertheless, from a practical point of view, it is clear that the codon context of specific genes can have adverse effects on both the quantity and quality of protein levels. Usually, this problem can be rectified by the alteration of the codons in question, or by the coexpression of the cognate tRNA genes.

PROTEIN DEGRADATION

Proteolysis is a selective, highly regulated process that plays an important role in cellular physiology (200, 203, 378). *E. coli* contains a large number of proteases that are localized in the cytoplasm, the periplasm, and the inner and outer membranes (25, 199, 201, 212, 367). These proteolytic enzymes participate in a host of metabolic activities, including the selective removal of abnormal proteins (201, 212). Protein damage or alteration may result from a variety of conditions, such as incomplete polypeptides, mutations caused by amino acid substitutions, excessive synthesis of subunits from multimeric complexes, posttranslational damage through oxidation or free-radical attack, and genetic engineering (201). Such abnormal proteins are efficiently removed by the bacterial proteolytic machine. To date, the mechanisms of protein degradation are incompletely understood, and it is unlikely that all proteolytic pathways or enzymes operating in *E. coli* have been identified yet. For example, a new protease associated with the outer membrane

TABLE 3—Continued

Detection	Applications	References
Antibody	Purification, detection	63, 259, 313, 446, 540
Antibody	Purification, detection	251, 252, 578, 619
Biochemical assay, antibody	Expression, purification, detection	101, 167, 225, 226, 462, 524
	Expression, purification, detection	411, 477, 568
Antibody, fluorescent calmodulin ligand	Expression, purification, detection	148, 230, 329, 418
	Purification, detection	403
Antibody	Expression, purification	330, 351, 591a
Biochemical assay, antibody	Expression, purification, detection	181, 182, 192, 278, 472, 518, 569
Antibody	Expression	19, 76, 319, 595
Biochemical assay	Purification	144, 166, 315, 459
	Purification, detection	307, 308
	Purification	596
Labeled biotin	Expression, purification	109
Antibody	Detection, purification	114, 608
	Purification, detection	6
	Purification, detection, assay systems	484, 485
Antibody	Detection, purification	410, 501
	Purification, detection	392, 584, 585
	Purification	281
	Expression	146
	Purification, refolding	61, 487, 488, 533, 534
	Purification	436
	Purification	436
	Purification, screening peptide libraries	115, 177, 347, 354, 491
	Expression	215
	Expression	176, 271, 365, 380
	Purification	34, 136, 358
	Purification	555
	Purification	244
	Purification, enzyme immobilization	431, 432
	Secretion into culture medium	303, 357, 528
		398–400
	Expression	267, 611
	In vitro phosphorylation, purification	88, 300
	Purification	318
Antibody	Purification, reverse epitope tagging ^c	557
	Detection, purification	582
UV light	Detection	81,113, 241

^a In addition to their utility in purification and detection, specific fusion peptides may confer advantages to the target protein during expression, such as increased solubility, protection from proteolysis, improved folding, increased yield, and secretion. These advantages are denoted as Expression in the Applications column. The engineering of specific protease sites in many fusion proteins facilitates the cleavage and removal of the fusion partner(s).

^b TPEG, *p*-aminophenyl- β -D-thiogalactoside.

^c CKS, CTP: CMP-3-deoxy-D-manno-octulosonate cytidyltransferase.

^d HAI, influenza virus hemagglutinin.

^e Reverse epitope tagging refers to tagging of the chromosomal rather than the plasmid-encoded protein, to avoid the need to remove the fusion partner.

use of chaperones for gene expression and provided detailed and rigorous assessments. This section is a distillation of the take-home lessons.

Normally, protein folding proceeds toward a thermodynamically stable end product (434, 476). Proteins that are drastically destabilized will probably fold incorrectly, even in the presence of chaperones. Thus, the truncation of polypeptides, the production of single domains from multisubunit protein complexes, the lack of formation of disulfide bonds which ordinarily contribute to protein structure (320, 559), or the absence of posttranslational modifications such as glycosylation (116) may make it impossible to attain thermodynamic stability. Moreover, it is now clear that different types of chaperones normally act in concert (69, 327). Therefore, the overproduction of a single chaperone may be ineffective. For example, the overproduction of DnaK alone resulted in plasmid

instability which was alleviated by the coproduction of DnaJ (52). Similarly, the coexpression of three chaperone genes in *E. coli* increased the solubility of several kinases (79). In some cases, it may be necessary to coexpress chaperones cloned from the same source as the target protein (105). Still another variable to consider is growth temperature. For example, GroES-GroEL coexpression increased the production of β -galactosidase at 30 but not 37 or 42°C, whereas DnaK and DnaJ were effective at all temperatures tested (180). Finally, the overexpression of chaperones can lead to phenotypic changes, such as cell filamentation, that can be detrimental to cell viability and protein production (52).

Two recent reports have shown that the coexpression of the human (268) or rat (433a) protein disulfide isomerase (PDI) with the target gene enhances the yield of correctly folded protein in the *E. coli* periplasm. Disulfide bond formation in

TABLE 3. Fusion partners and their applications^a

Fusion partner	Ligand/matrix	Purification conditions
Flag peptide	Anti-Flag monoclonal antibodies, M1, M2	Low calcium, EDTA, glycine
His ₆	Ni ²⁺ -nitrilotriacetic acid	Imidazole
Glutathione-S-transferase	Glutathione-Sepharose	Reduced glutathione
Staphylococcal protein A	Immunoglobulin G-Sepharose	Low pH, IgG-affinity ligand
Streptococcal protein G	Albumin	Low pH, albumin-affinity ligand
Calmodulin	Organic ligands, peptide ligands, DEAE-Sephadex	Low calcium
Thioredoxin	ThioBond resin	Ion exchange
β -Galactosidase	TPEG ^b -Sepharose	Borate
Ubiquitin		
Chloramphenicol acetyltransferase	Chloramphenicol-Sepharose	Chloramphenicol
S-peptide (RNase A, residues 1-20)	S-protein (RNase A, residues 21-124)	Denaturing or nondenaturing conditions
Myosin heavy chain		Differential solubility in low/high salt
DsbA		
Biotin subunit (in vivo biotinylation)		
Avidin	Biotin	Denaturation (urea, heat)
Streptavidin	Biotin	Denaturation (urea, heat)
Strep-tag	Streptavidin	2-Iminobiotin, diaminobiotin
c-myc	Anti-myc antibody	
Dihydrofolate reductase	Methotrexate-agarose	Folate buffer
CKS ^c		
Polyarginine	S-Sepharose	NaCl
Polycysteine	Thiopropyl-Sepharose	Dithiothreitol
Polyphenylalanine	Phenyl-Superose	Ethylene glycol
<i>lac</i> repressor	<i>lac</i> operator	Lactose analog, DNase, restriction endonuclease
T4 gp55		
Growth hormone N terminus		
Maltose-binding protein	Amylose resin	Maltose
Galactose-binding protein	Galactose-Sepharose	Galactose
Cyclomaltodextrin glucanotransferase	α -Cyclodextrin-agarose	α -Cyclodextrin
Cellulose-binding domain	Cellulose	Water
Hemolysin A, <i>E. coli</i>		
λ cII protein		
TrpE or TrpLE		
Protein kinase site(s)		
(AlaTrpTrpPro) _n		
HA1 ^d epitope		Aqueous two-phase extraction
BTag (VP7 protein region of bluetongue virus)	Anti-BTag antibodies	
Green fluorescent protein		

The fusion of genes to the ubiquitin sequence increased the yield of proteins from undetectable to 20% of the total cellular protein (76, 595). Similar results have been obtained by many other workers (reference 319 and references therein). The remarkable increase in protein yield was thought to be due to protection of the target protein from proteolysis, improved folding, and efficient mRNA translation (76). Ubiquitin or the ubiquitin metabolic pathway is absent in prokaryotic organisms. To remove the ubiquitin moiety from fusion proteins, Baker et al. (19) coexpressed the ubiquitin-specific protease Ubp2 in *E. coli*, thus effecting the cotranslational cleavage of ubiquitin from the fusion protein.

MOLECULAR CHAPERONES

It is now well established that the efficient posttranslational folding of proteins, the assembly of polypeptides into oligomeric structures, and the localization of proteins are mediated by specialized proteins termed molecular chaperones (33, 69, 104, 149, 183, 189, 246, 350, 364, 601). The demonstration that efficient production and assembly of prokaryotic ribulose biphosphate carboxylase in *E. coli* require both GroES and

GroEL proteins (208) led to an increasing interest in the use of molecular chaperones for high-level gene expression in *E. coli* (106). The experimental results from the use of chaperones, however, have been inconsistent, and thus far the effects of chaperone coproduction on gene expression in *E. coli* appear to be protein specific (581). For example, although the GroESL plasmids have been disseminated to more than 400 workers, only half of those who used them reported an improvement in gene expression (350a). This is consistent with recent observations that whereas the coproduction of thioredoxin in *E. coli* caused a dramatic increase in the solubility of eight vertebrate proteins, the coproduction of the GroESL chaperones increased the solubilities of only four of those proteins (613). It is also unclear whether the in vivo levels of different chaperone species are limiting under conditions of gene overexpression. For example, Knappik et al. (312) examined the effect of folding catalysts on the production of antibody fragments in the periplasm. Whereas the presence of the disulfide-forming protein DsbA was absolutely required in vivo, its overexpression did not increase the yield of antibody fragments. Wall and Plückthun (581) and Georgiou and Valax (180) revisited the assumptions and expectations behind the

TABLE 2—Continued

Compartment property	Strategy for resolution	Reference(s)
Extracellular medium		
Advantages		
Least level of proteolysis		309
Purification is simpler (fewest protein types)		
Improved protein folding		
N-terminus authenticity		
Disadvantages		
No secretion usually	Fusions to normally secreted proteins	303, 528
	Coexpression of <i>kil</i> for permeabilization	296, 309
	Fusion to <i>ompF</i> gene components	396
	Use of <i>ompA</i> signal sequence	316
	Use of protein A signal sequence	256
	Coexpression of bacteriocin release protein	261
	Use of glycine and bacteriocin release protein	617
	Glycine supplement in medium	10, 13
	Fusion partners	384
Protein diluted, more difficult to purify	Expanded-bed adsorption	231
	Concentration, affinity chromatography	
Cell surface		
To date not useful for high-level gene expression. May facilitate vaccine development, drug screening, biocatalysis, protein-protein interactions, and other applications		85, 111, 169, 178, 179, 253, 345, 352, 405

profound consequences. For example, the retention of the initiating methionine in RANTES, a member of the chemokine family of cytokines, completely abrogates the physiological activity of this molecule and confers potent antagonist properties to the methionylated RANTES (448). Similarly, an unnatural N-terminal methionine residue can alter the conformation of the human hemoglobin molecule (298). Moreover, it is possible that the presence of an extra amino acid will change the immunological properties of pharmaceutical proteins and create difficulties in the approval of a nonnative product for clinical use.

Bacterial translation is initiated by *N*-formylmethionine which is deformed during synthesis (2) but not necessarily removed. The N-terminal methionine might be cleaved off by an endogenous methionine aminopeptidase (39) depending on the side chain length of the second amino acid residue (250). Thus, residues with small side chains such as Gly, Ala, Pro, Ser, Thr, Val, Cys, and, to a lesser degree, Asn, Asp, Leu, and Ile, facilitate the methionine aminopeptidase-catalyzed removal of the N-terminal methionine (250). One strategy that has been successfully used to remove the extra methionine residue from recombinant proteins in vivo is coexpression of the *E. coli* methionine aminopeptidase gene (483, 513). An alternative method for the in vitro generation of an authentic N terminus uses the exopeptidase dipeptidylaminopeptidase I. This enzyme removes dipeptides from the N terminus but cannot cleave peptide bonds containing a proline residue. Dalbøge et al. (117) produced human growth hormone containing an amino-terminal extension which was subsequently removed with dipeptidylaminopeptidase I to yield authentic growth hormone. This approach requires an amino-terminal extension that contains an even number of amino acid residues and is designed so that it enables the in vivo excision of the N-terminal methionine. In addition, the second or third amino acid residue in the target protein must be proline (117). A more elaborate method free of the above restrictions has been

proposed to generate an authentic N terminus for any protein (117). The cotranslational amino-terminal processing in both prokaryotes and eukaryotes has been reviewed (301).

Protein degradation is more likely to occur in the cytoplasm of *E. coli* than in other compartments (550) because of the greater number of proteases located there (545, 546). This topic is examined in the section on protein degradation (below). Finally, another difficulty that affects cytosolic gene expression is the need to purify the target protein from the pool of the intracellular proteins. Calculations based on total DNA content predict that the *E. coli* chromosome may encode 3,000 to 4,000 genes (547), although not all of these are expressed under given growth conditions.

Periplasmic Expression

The periplasm offers several advantages for protein targeting. In contrast to the cytosolic compartment, the periplasm contains only 4% of the total cell protein (416) or approximately 100 proteins (450). The target protein is thus effectively concentrated, and its purification is considerably less onerous. The oxidizing environment of the periplasm facilitates the proper folding of proteins, and the cleaving in vivo of the signal peptide during translocation to the periplasm is more likely to yield the authentic N terminus of the target protein. Protein degradation in the periplasm is also less extensive (550).

The transport of a protein through the inner membrane to the periplasm normally requires a signal sequence (376, 490, 492, 575–577, 589). A wide variety of signal peptides have been used successfully in *E. coli* for protein translocation to the periplasm. These include prokaryotic signal sequences, such as the *E. coli* PhoA signal (127, 424), *OmpA* (127, 185, 205, 263, 339), *OmpT* (286), *LamB* and *OmpF* (255), β -lactamase (292, 574), enterotoxins ST-II, LT-A, LT-B (171, 388), protein A from *Staphylococcus aureus* (1, 256), endoglucanase from *B. subtilis* (348), *PeiB* from *Erwinia carotovora* (44, 340), a degen-

TABLE 2. Relative merits of different compartments for gene expression in *E. coli* and strategies for the potential resolution of experimental problems

Compartment property	Strategy for resolution	Reference(s)
Cytoplasm		
Advantages		25, 99, 243, 294, 310, 469
Inclusion bodies: facile isolation of protein in high purity and concentration; target protein protected from proteases; desirable for production of proteins that, if active, are lethal to host cell		
Higher protein yields		
Simpler plasmid constructs		
Disadvantages		
Inclusion bodies: protein insolubility; refolding to regain protein activity; refolded protein may not regain its biological activity; reduction in final protein yield; increase in cost of goods	Lower growth temperature Cold shock promoters (lower temperature) Selection of different <i>E. coli</i> strains Amino acid substitutions Coexpression of molecular chaperones Fusion partners Strains deficient in thioredoxin reductase Sorbitol and glycyl betaine in culture medium Altered pH Sucrose, raffinose in growth medium Rich growth media Strains deficient in thioredoxin reductase	495 187, 206, 433 302 118, 282, 394, 457, 536 119, 581, 613 330, 419, 591a 128, 447 49 541 56 386 128, 447
Reducing environment: does not facilitate disulfide bond formation		
Authenticity: N-terminal methionine	Coexpression of methionine aminopeptidase	483, 513
Proteolysis	Protease-deficient strains Mutagenesis of protease cleavage sites Hydrophobicity engineering Fusion partners Fermentation conditions Coexpression of phage T4 <i>pin</i> gene Coexpression of molecular chaperones Fusion of multiple copies of target gene Affinity fusion partners (may require cleavage)	211 25, 243, 395 394 59, 319, 393, 395, 567 24, 25, 100, 337 519-521 180, 489, 581 512 419
Purification is more complex (more protein types)		
Periplasm		
Advantages		
Purification is simpler (fewer protein types)		416
Proteolysis is less extensive	Protease-deficient strains Fusion partners Other approaches as above	372, 373 230
Improved disulfide bond formation/folding		
N-terminus authenticity		
Disadvantages		
Signal peptide does not always facilitate transport: protein export machinery overloaded?	Coexpression of signal peptidase I Co-overexpression of <i>prfF</i> Use of <i>prfF</i> mutant strains Coexpression of <i>prfA4</i> and <i>secE</i> Expression of <i>pspA</i> Coexpression of <i>sec</i> genes Fusion proteins	570 379 525 435 311 581 220
Reduced folding	Amino acid substitutions Coexpression of protein disulfide isomerase	314 268, 433a
Inclusion bodies may form	Coexpression of molecular chaperones Lower growth temperature Sucrose, raffinose in growth medium	45 57, 58, 82 56
Inner membrane		
To date not useful for high-level gene expression: may facilitate pharmacological studies, enzymatic activity studies, and other applications		220, 500

Continued on following page

universal mRNA stabilizer. For example, the replacement of the 3'-terminal hairpins of labile mRNAs with those from stable mRNAs did not enhance the expression of the labile transcripts (38, 89, 597). Furthermore, it has been suggested that gene expression might be enhanced by the use of host strains that are deficient in specific RNases, such as RNase II or PNPase. This, too, is unlikely to be an effective strategy, because the absence of RNase II or PNPase, as well as the overproduction of RNase II, had no effect on the average half-life of *E. coli* bulk mRNA (138, 139). Moreover, strains that were deficient in both RNase II and PNPase were inviable (138). These and other considerations led to the following conclusions: "It is unlikely that the disparate stabilities of most mRNAs that end in a stem-loop result from differential susceptibility of these terminal stem-loops to penetration by 3' exonucleases," and, furthermore, "3'-exonucleolytic initiation of RNA decay probably is rare, except in the case of labile RNAs lacking a substantial 3' hairpin and long-lived RNAs resistant to attack by all other types of ribonucleases" (35).

Translational Termination

The presence of a stop signal in the mRNA is an indispensable component of the translation termination process. In addition to the three termination codons, UAA, UGA, and UAG, this complex event involves specific interactions between the ribosome, mRNA, and several release factors at the site of termination (112, 553). In *E. coli*, RF-1 terminates translation at the UAG stop codon, RF-2 terminates translation at the UGA codon, and both RFs terminate translation at the UAA codon (507). An additional factor, RF-3, has recently been cloned (219, 377).

The design of expression vectors frequently includes the insertion of all three stop codons to prevent possible ribosome skipping. In *E. coli*, there is a preference for the UAA stop codon (508). A statistical analysis of more than 2,000 *E. coli* genes revealed local nonrandomness both in the stop codon and in the nucleotide immediately following the triplet (445, 553). The same workers tested the strengths of each of 12 possible tetranucleotide "stop signals" (UAAN, UGAN, UAGN) by an in vivo termination assay that measured termination efficiency by its direct competition with frameshifting. Termination efficiencies varied significantly depending on both the stop codon and the fourth nucleotide, ranging from 80% (UAAU) to 7% (UGAC). These findings indicate that the identity of the nucleotide immediately following the stop codon strongly influences the efficiency of translational termination in *E. coli* (445). Therefore, UAAU is the most efficient translational termination sequence in *E. coli*.

The sequence context at the 5' end of the stop codon further influences the efficiency of termination. Thus, the charge and hydrophobicity properties of the penultimate (-2 location) C-terminal amino acid residue in the nascent peptide cause up to a 30-fold difference in UGA termination efficiency, whereas termination at UAG is less sensitive to the nature of the -2 amino acid residue (389). For the -1 location, α -helical, β -strand, and reverse-turn propensities are determining factors in UGA termination (48).

PROTEIN TARGETING

Cytoplasmic Expression

The formation of inclusion bodies remains a significant barrier to gene expression in the cytosol. Inclusion bodies do offer several advantages (Table 2). However, these are small conso-

lation considering the arduous task of refolding the aggregated protein (469), the uncertainty of whether the refolded protein retained its biological activity, and the reduction in yield of the refolded and purified protein. To date, the precise physicochemical parameters that contribute to the formation of inclusion bodies remain unclear (322, 363, 381, 469, 495, 588). A statistical analysis of the composition of 81 proteins that do and do not form inclusion bodies in *E. coli* concluded that six parameters are correlated with inclusion body formation: charge average, turn-forming residue fraction, cysteine fraction, proline fraction, hydrophilicity, and total number of residues (591). The first two parameters are strongly correlated with inclusion body formation, while the last four parameters show a weak correlation. These findings were used to develop a model to predict the probability of inclusion body formation solely on the basis of the amino acid composition of a protein (591). This model was used to predict accurately the insolubility of the human T-cell receptor V β 5.3 in *E. coli* (9).

Several experimental approaches have been used to minimize the formation of inclusion bodies and improve protein folding (496) (Table 2). These include the growth of bacterial cultures at lower temperatures (77, 495, 497, 517); the selection of different *E. coli* strains (302); the substitution of selected amino acid residues (118, 457); the coproduction of chaperones (8, 29, 52, 337, 613); the use of *E. coli* thioredoxin either as a fusion partner (330) or coproduced with the protein of interest (613); growth and induction of the cells under osmotic stress in the presence of sorbitol and glycyl betaine (49); addition of nonmetabolizable sugars to the growth medium (56); alteration of the pH of the culture medium (541); and the use of strains deficient in thioredoxin reductase (128, 447).

The reducing potential of the cytoplasmic redox state (156, 270) presents still another problem. Bacterial cytoplasmic proteins contain few cysteine residues and few disulfide bonds (156, 444). Most proteins that contain stable disulfide bonds are exported from the cytoplasm (559). Thus, mammalian proteins whose complex tertiary structure depends in part on disulfide bond formation may not be produced in their correct conformation in the bacterial cytoplasm (443). Bardwell et al. have proposed that the low frequency of disulfide bonds in cytoplasmic proteins may be due to the absence from the cytoplasm of a system for the formation of disulfide bonds, such as the DsbA and DsbB proteins (26, 27), and/or a mechanism that actively prevents the formation of disulfide bonds in the cytoplasm. Mutant *E. coli* strains that allow the formation of disulfide bonds in the cytoplasm were isolated (128). These mutations inactivate the *trxB* gene that encodes thioredoxin reductase (168) and contributes to the sulfhydryl reducing potential of the cytoplasm (258). Thioredoxin itself was unnecessary for disulfide bond formation (128). The precise sequence of events is not clearly understood, and the authors suggested that the cytoplasm may contain another thioredoxin-like protein that can be reduced by thioredoxin reductase; in the absence of thioredoxin reductase, the oxidized form of this unknown protein facilitates the formation of disulfide bonds in the cytoplasm (128). Other workers have recently used *E. coli* strains carrying null mutations in the *trxB* gene and observed significant amounts of functional disulfide-containing protein in the cytoplasm (447). These thioredoxin reductase-deficient strains should prove to be valuable tools for the production of complex proteins in *E. coli*.

The cytoplasmic expression of a gene without a leader requires the presence of an initiation codon, the most common one encoding methionine. Although this extraneous amino acid might have no adverse effect on the protein synthesized, there are specific cases in which the extra methionine has

involved during the initiation of translation. Cross-linking studies, for example, have shown that the ribosomal protein S1 is directly involved in recognition and binding of mRNA by the 30S ribosomal subunit (54). The structural and functional interactions of the many components of the prokaryotic translational initiation complex have been examined (160, 224, 321, 368, 369, 579).

Translational Enhancers

Sequences that markedly enhance the expression of heterologous genes in *E. coli* have been identified in both bacteria and phages. Olins et al. characterized a 9-base sequence from the T7 phage gene 10 leader (g10-L) that appears to act as a very efficient RBS. Compared with a consensus SD region, the g10-L sequence caused a 40- to 340-fold increase in the expression of several genes (425, 428). When placed upstream of a synthetic SD sequence, the g10-L sequence caused a 110-fold increase in the translational efficiency of the *lacZ* gene, estimated as the ratio of β -galactosidase activity to the level of *lacZ* mRNA (427). A model was proposed whereby this sequence functions to enhance translation by interacting with bases 458 to 466 of the 16S rRNA (427). An alternative explanation is that only mRNAs with a weak SD site are likely to benefit from the g10-L sequence and that this might be due to stabilization of the mRNA rather than to a specific interaction with the 16S rRNA (527). Others failed to observe a significant enhancement of protein production when using the g10-L sequence (9, 527). Sequences homologous to the T7 g10-L have also been identified in other bacteriophages (427).

Several other groups have identified U-rich sequences in the 5' untranslated region (UTR) of mRNAs that act as enhancers of translation. McCarthy et al. (370) characterized a region in the *E. coli atpE* gene, immediately upstream of the SD site. This 30-base sequence was used to overexpress the human interleukin-2 and interferon beta genes (371, 493, 494). A U_8 sequence upstream of the SD site in the *md* mRNA encoding RNase D (620) was shown to be essential for efficient translation of this mRNA (622). Deletion of this region severely decreased translation without affecting the level of *md* mRNA or the transcriptional start site (621). Boni and coworkers demonstrated that the target for similar sequences is the S1 protein of the 30S ribosomal subunit (54, 565).

In a very interesting study, Sprengart et al. (527) demonstrated that sequences immediately downstream of the start codon play an important role during translation initiation. A specific region, termed the downstream box (DB), located between positions +15 to +26 of the T7 gene 0.3 coding region (526) or between positions +9 and +21 of the T7 gene 10 coding region (527) functions as a translational enhancer. The DB region is complementary to 16S rRNA nucleotides 1469 to 1483, termed the anti-downstream box (ADB). Deletion of the DB abolished translational activity (526). Conversely, optimization of the complementarity between the DB and the ADB sequences resulted in the highest level of expression of the *dhfr* fusion gene (527). Interestingly, the DB was not functional when shifted upstream of the initiation codon to the position of the SD sequence. The DB is present in a number of *E. coli* and bacteriophage genes (158, 242, 279, 326, 397, 442, 511).

These findings demonstrate convincingly that in addition to the SD site and the start codon, other sequences in the mRNA are important for efficient translation. Although the precise mechanisms of the observed effects are not always clear, the few studies cited above indicate that efforts to overexpress genes may benefit from the use of "translational enhancer" modules.

mRNA Stability

The process of mRNA degradation provides a major control point of gene expression in virtually all organisms (467). Although the concept of mRNA and its lability was established 35 years ago (60, 222, 283), the detailed understanding of the mechanisms of mRNA decay has presented a considerable challenge for a number of reasons (35). However, in spite of the many perplexing questions surrounding this important biological process, progress has been impressive (36, 147, 323, 407, 437). This section will consider specific determinants of mRNA stability that may have practical applications in the high-level expression of genes in *E. coli*.

Several different RNases participate in mRNA degradation in *E. coli*, including endonucleases (RNase E, RNase K, and RNase III) and 3' exonucleases (RNase II and polynucleotide phosphorylase [PNPase]); no 5' exonuclease has been identified to date in prokaryotes (35). mRNA degradation is not effected randomly by nonspecific endonucleolytic cleavage, since there is no inverse correlation between mRNA length and half-life (90). Two classes of protective elements are known to stabilize mRNAs in *E. coli*. One class consists of sequences in the 5' UTRs of mRNAs (31), and the other class includes stem-loop structures from the 3' UTRs and intercistronic regions (249). Some of these elements act as stabilizers when fused to heterologous mRNAs but only under restricted conditions. For example, the 5' UTR of the bacteriophage T4 gene 32 increases the half-life of unstable mRNAs in *E. coli* but only in T4-infected cells (143, 210). The erythromycin resistance genes (*erm*) of gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* encode mRNAs that contain stabilizing elements in their 5' UTRs. However, stabilization of mRNAs by the *ermC* and *ermA* 5' UTRs is induced by an antibiotic that inhibits translation and causes ribosome stalling (32, 481, 482). Similarly, the stabilizing effect of the 5' region of the bacteriophage λ p_L on λ p_L -*trp* transcripts requires λ infection (606).

In contrast to the above examples, the 5' UTR of the *E. coli ompA* transcript prolongs the half-life of a number of heterologous mRNAs in *E. coli* under normal conditions of rapid cell growth (38, 96, 151, 152). Emory et al. showed that the presence of a stem-loop structure at or very near the extreme 5' terminus of the *ompA* 5' UTR is essential for its stabilizing effect. Furthermore, the half-life of a normally labile mRNA could be prolonged in *E. coli* by adding a hairpin structure at its 5' terminus (152). It was proposed that *E. coli* mRNAs beginning with a long single-stranded segment are preferentially targeted by an RNase that interacts with the 5' terminus before cleaving the mRNA at internal sites (152). It appears, therefore, that the addition of the *ompA* 5' stabilizer to heterologous genes might enhance gene expression in *E. coli*. However, it is possible that mRNAs which contain internal RNA-processing sites are not protected by the presence of the 5' stabilizer (31).

The other class of mRNA-protective elements consists of 3' UTR sequences that can form stem-loop structures, thereby blocking exonucleolytic degradation of the transcript from the 3' terminus (249). Wong and Chang (597, 598) identified such an element within the transcription terminator of the crystal protein gene of *Bacillus thuringiensis*. Fusion of this "positive retroregulator" to the 3' termini of the penicillinase (*penP*) gene of *Bacillus licheniformis* and the human interleukin-2 cDNA increased the half-life of the mRNAs and enhanced the production of the corresponding polypeptides in both *B. subtilis* and *E. coli*. However, as in the case of some of the 5' stabilizers, this 3' retroregulator (597) is unlikely to act as a

gene expression is based on the proposition that the rate of protein folding will be only slightly affected at about 15 to 20°C, whereas the rates of transcription and translation, being biochemical reactions, will be substantially decreased. This, in turn, will provide sufficient time for protein refolding, yielding active proteins and avoiding the formation of inactive protein aggregates, i.e., inclusion bodies, without reducing the final yield of the target protein (433). It would be interesting to compare the transcriptional activities of other promoters derived from cold shock genes (288, 402).

Other promoters that have been characterized recently (Table 1) possess attractive features and should provide additional options for high-level gene expression systems. For example, the pH promoter (102, 561) is very strong: recombinant proteins are produced at levels of up to 40 to 50% of the total cellular protein (480). This expression level, however, will probably vary for different genes, because protein synthesis depends on translational efficiency as well as promoter strength.

E. coli promoters are usually considered in terms of a core region composed of the -10 and -35 hexameric sequences including a 15- to 19-bp spacer between the two hexamers (344). However, it has been proposed that elements outside the core region stimulate promoter activity (134). Many studies have demonstrated that sequences upstream of the core promoter increase the rate of transcription initiation in vivo (172, 213, 264, 290, 618). Gourse and colleagues have shown that a DNA sequence, the UP element, located upstream of the -35 region of the *E. coli* rRNA promoter *rrnB* P1, stimulates transcription by a factor of 30 in vitro and in vivo (290, 453, 468). The UP element functions as an independent promoter module because when it is fused to other promoters such as *lacUV5*, it stimulates transcription (453, 468). Upstream activation in *E. coli* and other organisms has been reviewed in detail (110). The ability of the UP element to act as a transcriptional enhancer when fused to heterologous promoters may be of general utility in high-level expression systems.

Although the extraordinary strength of the rRNA promoters P1 and P2 is well documented (173, 414), these promoters have not been exploited for the high-level production of proteins in *E. coli*, mainly because their regulation is more difficult. The in vivo synthesis of rRNA is subject to growth rate control (213), and P1 and P2 are active during periods of rapid cell growth and are downregulated when cells are in the stationary phase of growth. Therefore, the rRNA promoters would be continuously active or "leaky" during the preinduction phase. In vivo P2 is the weaker, less inducible promoter in rapidly growing cells. However, when uncoupled from P1, the P2 promoter shows increased activity (up to 70% of that of P1) and becomes sensitive to the stringent response, indicating that in its native tandem context, P2 is partially occluded (173, 289). Brosius and Holy (66) inserted the *lac* operator sequence downstream of the *rrnB* rRNA P2 promoter and achieved repression of P2 in strains harboring the *lacI^q* gene. Transcriptional activity was measured by the production of chloramphenicol acetyltransferase and by the expression of the 4.5S RNA. However, the P2 construction was only half as active as the *tac* promoter, and furthermore, when the *rrnB* P1 promoter was placed upstream of the P2 promoter, transcriptional repression was incomplete (66).

It is tempting to speculate that rRNA promoters could be tightly regulated by using the concept of inverted promoters (see the section on tightly regulated expression systems, below). Thus, a rRNA promoter could be cloned upstream of the gene of interest but in the opposite transcriptional direction. The use of λ integration sites and a regulated λ integrase

would facilitate the inversion of the promoter for induction, and the presence of strong transcription terminators upstream of the highly active promoter would prevent destabilization of the vector during the preinduction phase.

Transcriptional Terminators

In prokaryotes, transcription termination is effected by two different types of mechanisms: Rho-dependent transcription termination depends on the hexameric protein rho, which causes the release of the nascent RNA transcript from the template. In contrast, rho-independent termination depends on signals encoded in the template, specifically, a region of dyad symmetry that encodes a hairpin or stem-loop structure in the nascent RNA and a second region that is rich in dA and dT and is located 4 to 9 bp distal to the dyadic sequence (83, 122, 439, 455, 456, 465, 594, 609). Although often overlooked in the construction of expression plasmids, efficient transcription terminators are indispensable elements of expression vectors, because they serve several important functions. Transcription through a promoter may inhibit its function, a phenomenon known as promoter occlusion (5). This interference can be prevented by the proper placement of a transcription terminator downstream of the coding sequence to prevent continued transcription through another promoter. Similarly, a transcription terminator placed upstream of the promoter that drives expression of the gene of interest minimizes background transcription (413). It is also known that transcription from strong promoters can destabilize plasmids as a result of overproduction of the ROP protein involved in the control of plasmid copy number as a result of transcriptional readthrough into the replication region (539). In addition, transcription terminators enhance mRNA stability (237, 404, 597) and can substantially increase the level of protein production (237, 572). Particularly effective are the two tandem transcription terminators T1 and T2, derived from the *rrnB* rRNA operon of *E. coli* (67), but many other sequences are also quite effective.

Transcriptional Antiterminators

In bacteria, many operons involved in amino acid biosynthesis contain transcriptional attenuators at the 5' end of the first structural gene. The attenuators are regulated by the amino acid products of the particular operon. Thus, the availability of the cognate charged tRNA leads to the formation of a secondary structure in the nascent transcript followed by ribosome stalling. In the absence of the cognate charged tRNA, an antiterminator structure which prevents formation of the RNA hairpin in the terminator and prevents transcriptional termination is formed (325). The antiterminator element that enables RNA polymerase to override a rho-dependent terminator in the ribosomal RNA operons has been identified and is referred to as *boxA* (41, 341). Transcriptional antitermination is a remarkably complex process that involves many known and as yet unidentified host factors. This topic has been covered in great detail in two excellent recent reviews (110, 456). Here, we will briefly consider the use of antitermination elements that are useful in the expression of heterologous genes in *E. coli*.

One of the more powerful and widely used expression systems in *E. coli* makes use of the phage T7 late promoter (537, 548). The activity of this system depends on a transcription unit that supplies the T7 RNA polymerase, whose tight repression is essential to avoid leakiness of the T7 promoter. Several approaches have been used to regulate the expression of the T7 polymerase, and each has its own unique disadvantages (374). Mertens et al. (374) addressed this problem by constructing a reversibly attenuated T7 RNA polymerase expres-

TABLE 1. Promoters used for the high-level expression of genes in *E. coli*

Promoter (source)	Regulation	Induction	Reference(s)
<i>lac</i> (<i>E. coli</i>)	<i>lacI</i> , <i>lacI</i> ^a <i>lacI</i> (Ts), ^a <i>lacI</i> ^a (Ts) ^a <i>lacI</i> (Ts) ^b	IPTG Thermal Thermal Trp starvation, indole acrylic acid IPTG, lactose ^c	17, 18, 221, 460, 610 234 604 365, 470, 549, 612 128a, 142, 185, 275, 401
<i>trp</i> (<i>E. coli</i>)		Nalidixic acid	84, 274, 291, 306, 382, 562
<i>lpp</i> (<i>E. coli</i>)	<i>phoB</i> (positive), <i>phoR</i> (negative)	Phosphate starvation	145, 260, 428, 516
<i>phoA</i> (<i>E. coli</i>)	<i>lexA</i>	L-Arabinose	554
<i>recA</i> (<i>E. coli</i>)	<i>araC</i>	Osmolarity	247
<i>araBAD</i> (<i>E. coli</i>)		Glucose starvation	564
<i>proU</i> (<i>E. coli</i>)		Tetracycline	125, 523
<i>cst-1</i> (<i>E. coli</i>)		pH	102, 480, 561
<i>tetA</i> (<i>E. coli</i>)	<i>cadR</i>	Anaerobic conditions, nitrate ion	335
<i>cadA</i> (<i>E. coli</i>)	<i>fnr</i> (FNR, NARL)		7, 123, 471
<i>nar</i> (<i>E. coli</i>)	<i>lacI</i> , <i>lacI</i> ^a	IPTG	603
<i>tac</i> , hybrid (<i>E. coli</i>)	<i>lacI</i> ^a	Thermal	65
	<i>lacI</i> , <i>lacI</i> ^a	IPTG	4, 9
<i>trc</i> , hybrid (<i>E. coli</i>)	<i>lacI</i> (Ts), ^a <i>lacI</i> ^a (Ts) ^a	Thermal	261, 263
	<i>lacI</i>	IPTG	186
<i>lpp-lac</i> , hybrid (<i>E. coli</i>)	<i>lacI</i> , <i>lacI</i> ^a	IPTG	366
<i>P_{syn}</i> , synthetic (<i>E. coli</i>)			
Starvation promoters (<i>E. coli</i>)			
<i>p_L</i> (λ)	λ Clis857	Thermal	43, 80, 129, 130, 240, 454
<i>p_L</i> -9G-50, mutant (λ)		Reduced temperature (<20°C)	187, 433
<i>cspA</i> (<i>E. coli</i>)		Reduced temperature (<20°C)	187, 206, 433, 551
<i>p_R</i> , <i>p_L</i> , tandem (λ)	λ Clis857	Thermal	150, 493
T7 (T7)	λ Clis857	Thermal	537, 548
T7- <i>lac</i> operator (T7)	<i>lacI</i> ^a	IPTG	141, 190, 239
λ p _L , p _{T7} , tandem (λ , T7)	λ Clis857, <i>lacI</i> ^a	Thermal, IPTG	375
T3- <i>lac</i> operator (T3)	<i>lacI</i> ^a	IPTG	190, 605
T5- <i>lac</i> operator (T5)	<i>lacI</i> ^a , <i>lacI</i>	IPTG	71, 390
T4 gene 32 (T4)		T4 infection	143, 210
<i>nprM-lac</i> operator (<i>Bacillus</i> spp.)	<i>lacI</i> ^a	IPTG	605
VHb (<i>Vitreoscilla</i> spp.)		Oxygen, cAMP-CAP ^e	304, 305
Protein A (<i>Staphylococcus aureus</i>)			1,256, 349

^a *lacI* gene with single mutation, Gly-187 → Ser (72).^b *lacI* gene with three mutations, Ala-241 → Thr, Gly-265 → Asp, and Ser-300 → Asn (604).^c The constitutive *lpp* promoter (*P_{lpp}*) was converted into an inducible promoter by insertion of the *lacUV5* promoter/operator region downstream of *P_{lpp}*. Thus, expression occurs only in the presence of a *lac* inducer (142).^d Wild-type *lacI* gene.^e cAMP-CAP, cyclic AMP-catabolite activator protein.

Lanzer and Bujard carried out extensive studies on the commonly used *lac*-based promoter-operator systems and demonstrated up to 70-fold differences in the level of repression when the operator was placed in different positions within the promoter sequence (328). Thus, when the 17-bp operator was placed between the -10 and -35 hexameric regions, a 50- to 70-fold-greater repression was caused than when the operator was placed either upstream of the -35 region or downstream of the -10 site (328).

A third important characteristic of a promoter is its inducibility in a simple and cost-effective manner. The most widely used promoters for large-scale protein production use thermal induction (λ *p_L*) or chemical inducers (*trp*) (Table 1). The isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible hybrid promoters *tac* (123) or *trc* (65) are powerful and widely used for basic research. However, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity (159) and cost. These drawbacks of IPTG have until now precluded the use of the *tac* or *trc* promoter from the production of human therapeutic proteins and rendered the large-scale expression of proteins for basic research prohibitively expensive. The availability of a mutant *lacI*(Ts) gene that encodes a thermosensitive *lac* repressor (72) now permits the thermal induction of these promoters (4, 9, 234). In addition, the new vectors exhibit tight regulation of the *trc*

promoter at 30°C (9). Two different *lac* repressor mutants that are thermosensitive (586, 604) as well as IPTG inducible (586) have recently been described. Although the wild-type *lacI* gene can be thermally induced (602, 603), this system is not tightly regulated and cannot be used in *lacI*^a strains, since a temperature shift does not override the tight repression caused by the overproduction of the *lac* repressor (603). Thus, this system is limited to the production of some proteins that are not detrimental to the host cell.

Cold-responsive promoters, although much less extensively studied than many of the other promoters included here, have been shown to facilitate efficient gene expression at reduced temperatures. The activity of the phage λ *p_L* promoter was highest at 20°C and declined as the temperature was raised (187). This cold response of the *p_L* promoter is positively regulated by the *E. coli* integration host factor, a sequence-specific, multifunctional protein that binds and bends DNA (164, 165, 188). The promoter of the major cold shock gene *cspA* (206, 551) was similarly demonstrated to be active at reduced temperatures (187). Molecular dissection of the *cspA* and *p_L* promoters led to the identification of specific DNA regions involved in the enhancement of transcription at lower temperatures: this has allowed the development of *p_L* derivatives that are highly active at temperatures below 20°C (433). The rationale behind the use of cold-responsive promoters for

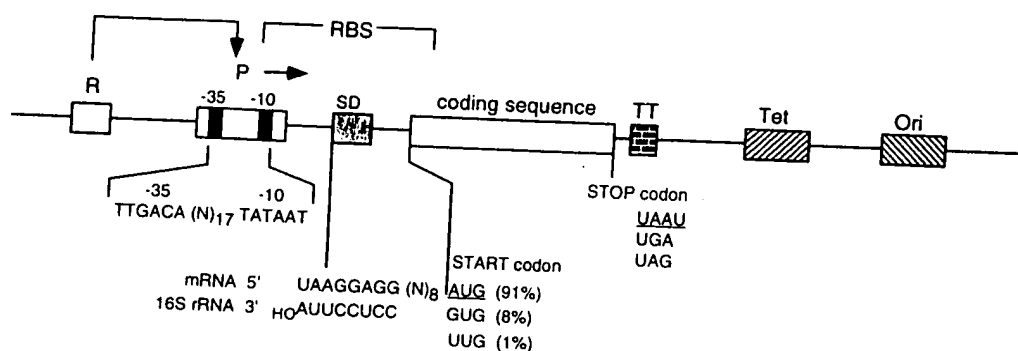


FIG. 1. Schematic presentation of the salient features and sequence elements of a prokaryotic expression vector. Shown as an example is the hybrid *tac* promoter (P) consisting of the -35 and -10 sequences, which are separated by a 17-base spacer. The arrow indicates the direction of transcription. The RBS consists of the SD sequence followed by an A+T-rich translational spacer that has an optimal length of approximately 8 bases. The SD sequence interacts with the 3' end of the 16S rRNA during translational initiation, as shown. The three start codons are shown, along with the frequency of their usage in *E. coli*. The repressor is encoded by a regulatory gene (R), which may be present on the vector itself or may be integrated in the host chromosome, and it modulates the activity of the promoter. The transcription terminator (TT) serves to stabilize the mRNA and the vector, as explained in the text. In addition, an antibiotic resistance gene, e.g., for tetracycline, facilitates phenotypic selection of the vector, and the origin of replication (Ori) determines the vector copy number. The various features are not drawn to scale.

CONFIGURATION OF EFFICIENT EXPRESSION VECTORS

The construction of an expression plasmid requires several elements whose configuration must be carefully considered to ensure the highest levels of protein synthesis (22, 64, 120, 142, 355, 538, 612). The essential architecture of an *E. coli* expression vector is shown in Fig. 1. The promoter is positioned approximately 10 to 100 bp upstream of the ribosome-binding site (RBS) and is under the control of a regulatory gene, which may be present on the vector itself or integrated in the host chromosome. Promoters of *E. coli* consist of a hexanucleotide sequence located approximately 35 bp upstream of the transcription initiation base (-35 region) separated by a short spacer from another hexanucleotide sequence (-10 region) (174, 232, 236, 344, 465). There are many promoters available for gene expression in *E. coli*, including those derived from gram-positive bacteria and bacteriophages (Table 1). A useful promoter exhibits several desirable features: it is strong, it has a low basal expression level (i.e., it is tightly regulated), it is easily transferable to other *E. coli* strains to facilitate testing of a large number of strains for protein yields, and its induction is simple and cost-effective (612).

Downstream of the promoter is the RBS, which spans a region of approximately 54 nucleotides bound by positions -35 (± 2) and +19 to +22 of the mRNA coding sequence (269). The Shine-Dalgarno (SD) site (514, 515) interacts with the 3' end of 16S rRNA during translation initiation (133, 532). The distance between the SD site and the start codon ranges from 5 to 13 bases (93), and the sequence of this region should eliminate the potential of secondary-structure formation in the mRNA transcript, which can reduce the efficiency of translation initiation (198, 229). Both 5' and 3' regions of the RBS exhibit a bias toward a high adenine content (140, 499, 502).

The transcription terminator is located downstream of the coding sequence and serves both as a signal to terminate transcription (465) and as a protective element composed of stem-loop structures, protecting the mRNA from exonucleolytic degradation and extending the mRNA half-life (35, 37, 147, 249, 597).

In addition to the above elements that have a direct impact on the efficiency of gene expression, vectors contain a gene that confers antibiotic resistance on the host to aid in plasmid selection and propagation. Ampicillin is commonly used for this purpose; however, for the production of human therapeutic

proteins, other antibiotic resistance markers are preferable to avoid the potential of human allergic reactions (42). Finally, the copy number of plasmids is determined by the origin of replication. In specific cases, the use of runaway replicons results in massive amplification of plasmid copy number concomitant with higher yields of plasmid-encoded protein (387, 415). In other cases, however, there appeared to be no advantage in using higher-copy-number plasmids over pBR322-based vectors (612). Furthermore, Vasquez et al. (572) reported that increasing the copy number of the plasmid decreased the production of trypsin in *E. coli* and Minas and Bailey (379) found that the presence of strong promoters on high-copy-number plasmids severely impaired cell viability.

TRANSCRIPTIONAL REGULATION

Promoters

A promoter for use in *E. coli* (Table 1) should have certain characteristics to render it suitable for high-level protein synthesis (207, 612). First, it must be strong, resulting in the accumulation of protein making up 10 to 30% or more of the total cellular protein.

Second, it should exhibit a minimal level of basal transcriptional activity. Large-scale gene expression preferably employs cell growth to high density and minimal promoter activity, followed by induction or derepression of the promoter. The tight regulation of a promoter is essential for the synthesis of proteins which may be detrimental to the host cell (see, e.g., references 68, 137, 544, 563, and 599). For example, the toxic rotavirus VP7 protein effectively kills cells and must be produced under tightly regulated conditions (592). However, in some cases, promoter stringency is inconsequential, because even the smallest amount of gene product drastically curtails bacterial survival because of its severe toxicity (615). For example, molecules that inactivate ribosomes or destroy the membrane potential would be lethal. Toxicity to the host is not restricted to foreign genes but may also result from the overexpression of certain native genes, such as the *trtA* gene, which encodes an outer membrane lipoprotein (423), the *EcoRI* restriction endonuclease in the absence of the corresponding protective *EcoRI* modification methylase (423), and the *lon* gene (558). Furthermore, incompletely repressed expression systems can cause plasmid instability, a decrease in cell growth rate, and loss of recombinant protein production (40, 98, 374).

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Strategies for Achieving High-Level Expression of Genes in *Escherichia coli*[†]

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INTRODUCTION

The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, and biological activity of the protein of interest, as well as regulatory issues in the production of therapeutic proteins (191, 254). In addition, the selection of a particular expression system requires a cost breakdown in terms of process, design, and other economic considerations. The relative merits of bacterial, yeast, insect, and mammalian expression systems have been examined in detail in an excellent review by Marino (362). In addition, Datar et al. (121) have analyzed the economic issues associated with protein production in bacterial and mammalian cells.

The many advantages of *Escherichia coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins (177a, 197, 254, 362, 406, 426, 510). However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. This may be due to the unique and subtle structural features of the gene sequence, the

stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host. Fortunately, some empirical "rules" that can guide the design of expression systems and limit the unpredictability of this operation in *E. coli* have emerged. The major drawbacks of *E. coli* as an expression system include the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulfide bond formation. On the other hand, many eukaryotic proteins retain their full biological activity in a nonglycosylated form and therefore can be produced in *E. coli* (see, e.g., references 170, 342, and 486). In addition, some progress has been made in the areas of extracellular secretion and disulfide bond formation, and these will be examined.

The objectives of this review are to integrate the extensive published literature on gene expression in *E. coli*, to focus on expression systems and experimental approaches useful for the overproduction of proteins, and to review recent progress in this field. Areas that have been covered in detail in recent reviews are included in abbreviated form in order to present their key conclusions and to serve as a source for further reading. As a matter of definition, the terms "periplasmic expression" and "extracellular secretion" will be used to refer to the targeting of protein to the periplasm and the culture medium, respectively, to avoid confusion.

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[†] This review is dedicated to the memory of William John Steele, an inspired scientist, a great man, mentor, and friend, who died on 8 December 1995. The world is a better place because of him.